VIROLOGY 211, 481–490 (1995)

Herpesvirus of Turkey Recombinant Viruses Expressing Infectious Bursal Disease Virus (IBDV) VP2 Immunogen Induce Protection against an IBDV Virulent Challenge in Chickens

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Received April 6, 1995; accepted June 9, 1995

Two recombinant herpesviruses of turkey (HVT) expressing the VP2 protein of infectious bursal disease virus (IBDV or Gumboro disease virus) have been constructed: vHVT001 and vHVT002. The VP2 open reading frame was inserted at the locus of the small subunit of ribonucleotide reductase gene (HSV-1 UL40 homolog) without any exogenous promoter in vHVT001 and at the locus of gI gene (HSV-1 US7 homolog) under the control of the human cytomegalovirus immediate-early promoter in vHVT002. The isolation of these recombinant viruses indicated that the deleted genes were not required for replication of HVT in chicken embryo fibroblasts. Efficacy of these recombinant viruses against IBDV strain 52/70 and Marek's disease virus (MDV strain RB1B) virulent challenges was evaluated in chickens vaccinated at 1 day of age. In the IBDV challenge, a good protection against mortality and bursal gross lesion was observed in vHVT002-vaccinated chickens: 100% with 10^5 PFU dose and 60% with 10^4 PFU dose; in contrast, only a weak level of protection was achieved after vaccination with vHVT001. Protection levels against MDV challenge obtained with vHVT001 and vHVT002 were low (around 10%) compared to that induced by the parental HVT (84%). In spite of the low protection level against MDV, this is the first report which describes induction of full protection against IBDV with a single inoculation of a recombinant virus.

INTRODUCTION

Infectious bursal disease virus (IBDV), the etiological agent of infectious bursal disease (or Gumboro disease), causes severe immunodepression of B cell response in young chickens by destruction of lymphocytes in the bursa of Fabricius. In chickens that are fully susceptible to IBDV infection, disease is responsible for losses due to impaired growth and death and to immunosuppression which increases susceptibility to other diseases and interferes with vaccination against Newcastle disease, Marek's disease, or infectious bronchitis (reviewed in Kibenge *et al.*, 1988).

IBDV is a member of the Birnaviridae family and its genome consists of two segments of double-stranded RNA (Dobos *et al.*, 1979). The smaller segment (segment B) encodes VP1, the putative double-strand RNA-dependent RNA polymerase (Azad *et al.*, 1985; Spies *et al.*, 1987). The larger segment (segment A) contains two open reading frames. One codes for VP5, recently identified (Mundt *et al.*, 1995). The second ORF codes for a polyprotein which is processed into three viral proteins, VP2, VP4, and VP3, by autoproteolysis (Azad *et al.*, 1987). VP2 and VP3 are the major structural proteins of the virion. VP3 contains two nonoverlapping epitopes recognized by monoclonal antibodies (Öppling *et al.*, 1991b). With the exception of one which neutralizes virus infection with a low titer (Reddy *et al.*, 1992), monoclonal antibodies directed against the VP3 protein do not neutralize IBDV infection (Becht *et al.*, 1988; Fahey *et al.*, 1991). VP2 is the major host-protective antigen of IBDV. It contains at least three independent epitopes responsible for the induction of neutralizing antibodies (Becht *et al.*, 1988; Fahey *et al.*, 1991). In addition, it has been demonstrated that this induction depends on the conformation of the antigenic site (Fahey *et al.*, 1989; Öppling *et al.*, 1991a).

Passive protection of chickens against IBDV is achieved by vaccinating breeding hens with conventional attenuated or inactivated IBDV vaccines. Maternal antibodies are transmitted to the progeny through the egg yolk and provide protection to chickens for the first few weeks of life. IBDV variant strains in the United States (Rosenberger and Cloud, 1985; Snyder *et al.*, 1988, 1992) and highly virulent strains in Europe (Van den Berg *et al.*, 1991) have been isolated from vaccinated flocks. These IBDV strains are able to escape from maternal antibodies induced by classical strains and cause economic losses in poultry industry. Because of the outcome of those new strains, broilers are now vaccinated at 2–3 weeks of age with "intermediate" strains (in the United States) or "hot" strains (in Europe). But since these strains

The nucleotide sequence data reported in this article have been deposited with the EMBL/GenBank Database under Accession No. L40429.

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0042-6822/95 $12.00

still retain some degree of pathogenicity, they induce reduced weight gain and may cause sometimes illness in chickens.

In recent years, different investigators have used the recombinant technology to express stuctural proteins of IBDV as subunit vaccines. VP2, expressed in *Escherichia coli,* induced high levels of anti-VP2 antibodies in chickens, but these antibodies did not neutralize virus infection and no passive protection against IBDV infection was observed (Azad *et al.,* 1991). However, passive protection of specific-pathogen-free (SPF) chickens against IBDV could be achieved using a VP2 recombinant protein expressed in yeast (Macreadie *et al.,* 1990). Chickens vaccinated with a recombinant fowlpox virus (rFPV) expressing VP2 fused to the β-galactosidase gene were protected against mortality but not against damage to the bursa of Fabricius (Bayliss *et al.,* 1991). Protection against bursal lesions was obtained using rFPV expressing a native form of the VP2 antigen but the level of protection was lower than that obtained with an inactivated vaccine (Heine and Boyle, 1993). Baculovirus expression system was also used to produce recombinant IBDV antigens in insect cells, and active and passive protection against virulent challenge were obtained using those antigens (Vakharia *et al.,* 1994).

Herpesvirus of turkey (HVT) has been used extensively as a vaccine against Marek's disease since 1971 (Witter, 1985), and previous studies have shown that HVT can be used as a vector for expressing antigens of Newcastle disease virus (Morgan *et al.,* 1992, 1993). In this report we describe the construction of two recombinant HVT viruses expressing the VP2 protein of IBDV. These recombinant HVT viruses were tested for their abilities to protect chickens against IBDV or Marek's disease virus (MDV) virulent challenges.

MATERIALS AND METHODS

Chickens

One-day-old SPF white leghorn chickens were obtained from Lohman (Cuxhaven, Germany).

Cells and viruses

The FC126 strain of HVT (Witter *et al.,* 1970) was propagated in chicken embryo fibroblasts (CEF) as described previously (Ross *et al.,* 1975). The highly oncogenic RB1B strain of MDV (Schat *et al.,* 1982) was used as the challenge virus for Marek's disease. The 52/70 strain of IBDV (Bygrave and Faragher, 1970) was propagated in SPF chickens. Infected bursae collected from these chickens were grinded to constitute the virus stock for the Gumboro disease challenge. This stock was titered on SPF embryonated eggs.

HVT DNA preparations

DNA for cloning was extracted from purified HVT nucleocapsids using proteinase K and SDS as described previously (Lee *et al.,* 1980). For transfection, total HVT-infected cell DNA was extracted as described previously (Morgan *et al.,* 1990).

Synthesis and cloning of IBDV VP2 gene

IBDV virions were purified from bursae of Fabricius of chickens infected with the 52/70 strain, following the method described by Azad (Azad *et al.,* 1985). Genomic RNA was extracted from purified virions treated with SDS (0.1%) and proteinase K (100 μg/ml) and was recovered after phenol/chloroform extraction by ethanol precipitation. cDNAs were synthesized as described previously (Azad *et al.,* 1985). Briefly, the IBDV double-stranded RNA solution was heated at 100° for 15 min and then frozen. After the RNA solution thawed, first-strand cDNA was synthesized by random priming using random hexamer primers and the Rous-associated virus reverse transcriptase (Amersham). Second-strand synthesis was carried out as described previously (Gubler and Hoffman, 1983). All cloning procedures were done using standard methods (Sambrook *et al., 1989). A* 3.1-kbp *Xbal–Kpnl* cDNA fragment corresponding to the larger RNA segment (segment A) of IBDV was cloned into vector pBluescript(SK$^+$) (pBS(SK$^+$); Stratagene, La Jolla, CA) to generate plasmid plBDVA00 (Fig. 1C). This plasmid contains the entire sequence coding for the polyprotein VP2–VP4–VP3. The sequence of IBDV 52/70 strain segment A has been described by Bayliss *et al.* (1990). A direct mutagenesis (Kunkel, 1985) was done on plasmid plBDVA00 with oligonucleotide GB010 5′ AAGGAGGTAAGCTTTGCCGGTG 3′ to introduce a TAA stop codon in the polyprotein reading frame at the putative cleavage site between amino acids 453 and 454 (Bayliss *et al.,* 1990) and a *Hind*III site right behind this stop codon. This plasmid, containing an open reading frame of 453 amino acids (VP2), was designated plBDVA01 (Fig. 1C).

Plasmid constructions for ribonucleotide reductase (RR2) deletion (pGH010)

The *Bam*HI-K1 fragment (Fig. 1A) of HVT DNA (Igarashi *et al.,* 1987) was cloned into vector pUC18 to generate plasmid pHVTK1 (Fig. 1B). This plasmid contains a 1.3-kbp *Bam*HI–*Eco*RI fragment and a 2.0-kbp *Xmn*I–*Bam*HI fragment which were subcloned as follows. The 1.3-kbp *Bam*HI–*Eco*RI fragment was isolated from pHVTK1 and cloned into vector pBS(SK$^+$), previously digested with *Bam*HI and *Eco*RI, to generate plasmid pHVT5. A direct mutagenesis was done on plasmid pHVT5 with oligonucleotide RR001 5′ CATGGCGGCGATCGTGAGTTA 3′ to introduce a *Pvu*I site 6 bp upstream from the RR2 start codon. This plasmid was designated pHVT5P (Fig. 1B).

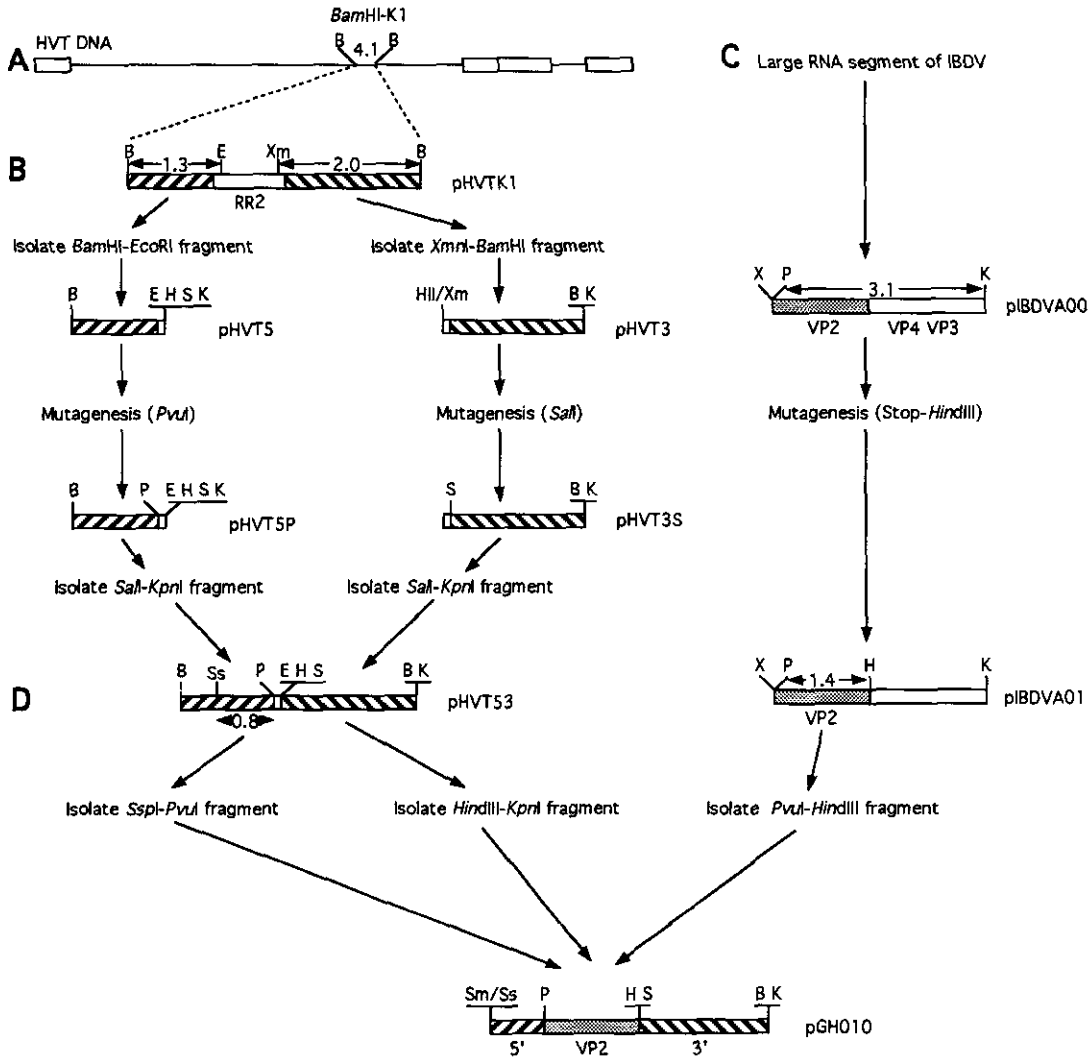

FIG. 1. Construction of RR2 deletion donor plasmid (pGH010). (A) Structure of the HVT genome showing location of the 4.1-kbp *Bam*HI-K1 fragment. (B) Structure of the *Bam*HI-K1 fragment showing the RR2 open reading frame and flanking sequences, and construction of plasmid pHVT53. (C) Cloning of the IBDV VP2 gene. (D) Construction and structure of donor plasmid pGH010 showing 5′ and 3′ HVT RR2 flanking sequences (hatched bars) surrounding the IBDV VP2 open reading frame (shaded bar). For details see Materials and Methods. Fragment sizes are in kbp. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; HII, *Hin*dII; K, *Kpn*I; P, *Pvu*I; S, *Sal*I; Sm, *Sma*I; Ss, *Ssp*I; X, *Xba*I; Xm, *Xmn*I; HII/Xm, ligation between *Hin*dII and *Xmn*I.

The 2.0-kbp *Xmn*I–*Bam*HI fragment was isolated from pHVTK1 and cloned into M13mp19 previously digested with *Hin*dII and *Bam*HI to generate pHVT3. A direct mutagenesis was done on pHVT3 with the oligonucleotide RR002 5′ CCGATTCACGTCGACTTTAGG 3′ to introduce a *Sal*I site 26 bp downstream from the RR2 stop codon and 11 bp upstream from the RR2 polyadenylation signal, to generate pHVT3S (Fig. 1B). The 1.9-kbp *Sal*I–*Kpn*I fragment was isolated from pHVT3S and cloned into pHVT5P, previously digested with *Sal*I and *Kpn*I, to generate plasmid pHVT53 (Fig. 1D). Plasmid pHVT53 was digested with *Ssp*I and *Pvu*I or *Hin*dIII and *Kpn*I to isolate respectively a 0.8-kbp *Ssp*I–*Pvu*I fragment (fragment A) and a 1.9-kbp *Hin*dIII–*Kpn*I fragment (fragment B). Plasmid pIBDVA01 was digested with *Pvu*I and *Hin*dIII to isolate a 1.4-kbp *Pvu*I–*Hin*dIII fragment (fragment C). Fragments A, B, and C were ligated together into pBS(SK$^+$), previously digested with *Sma*I and *Kpn*I, to generate donor plasmid pGH010. This plasmid contains the 5′ and 3′ HVT RR2 flanking sequences surrounding the VP2 gene of IBDV.

Plasmid constructions for gI deletion (pEL025)

The *Bam*HI A fragment (Fig. 2A) of HVT DNA (Igarashi *et al.*, 1987) was cloned into vector pBR322 to generate plasmid pRD001. The *Bam*HI-A fragment (pRD001) was subcloned into pBR322 as *Pst*I fragments to generate plasmids pRD006 (2.8-kbp *Pst*I subfragment) and pRD007 (5.7-kbp *Pst*I subfragment) (Fig. 2B). Plasmid pRD006 was digested with *Sac*I and *Pst*I to isolate a 0.3-kbp *Sac*I–*Pst*I fragment (fragment D). A PCR was done using primers pBR322-*Pst*I(−) (Boehringer Mannheim,

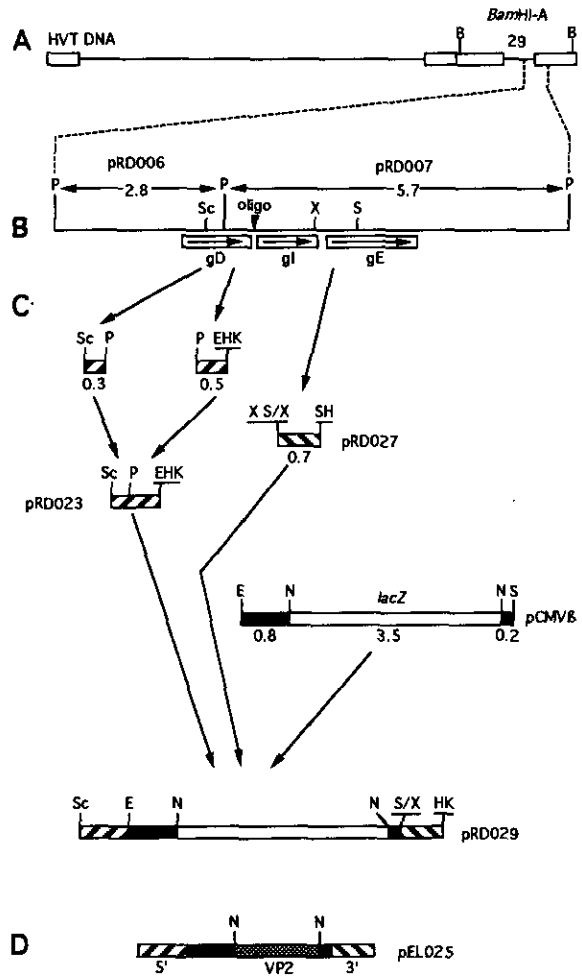

FIG. 2. Construction of gI deletion donor plasmid (pEL025). (A) Structure of the HVT genome showing location of the 29-kbp *Bam*HI-A fragment. (B) Structure of the two *Pst*I subclones, pRD006 and pRD007, used for donor plasmid construction showing sites of interest and oligonucleotide position. Map of encoded open reading frames in the region of interest from Zelnik *et al.* (1993). (C) Construction of pRD029. (D) Structure of donor plasmid pEL025. For details see Materials and Methods. Hatched bars, 5′ and 3′ HVT gI flanking regions; closed bars, HCMV IE promoter (0.8 kbp) and SV40 late gene polyadenylation signal (0.2 kbp); open bars, *lacZ* gene; shaded bar, IBDV VP2 open reading frame. Fragment sizes are in kbp. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Not*I; P, *Pst*I; S, *Sal*I; Sc, *Sac*I; X, *Xho*I; S/X, ligation between *Sal*I and *Xho*I.

Germany) and RD045 and plasmid pRD007 as template. Primer RD045 (5′ TGCTGGTACCGTCGACAAGCTTAGCTGAATTCAGCTGGATCCGTGCAGATAACACGTACTGGC 3′) was used to introduce a multiple cloning site 10 bp upstream from the gI start codon (position 6991; Zelnik *et al.*, 1993). The 0.5-kbp PCR fragment was then digested with *Pst*I and *Kpn*I and cloned with fragment D into pBS(SK$^+$) previously digested with *Sac*I and *Kpn*I. The resultant plasmid was designated pRD023 (Fig. 2C). The 0.7-kbp *Xho*I–*Sal*I fragment was isolated from pRD007 and cloned into pBS(SK$^+$) previously digested with *Sal*I and treated with alkaline phosphatase (Boehringer), to generate plasmid pRD027 (Fig. 2C). Plasmid pRD027 was digested with *Xho*I and *Hin*dIII to isolate a 0.7-kbp *Xho*I–*Hin*dIII fragment (fragment E). Plasmid pCMVβ (Clontech, Palo Alto, CA) was digested with *Eco*RI and *Sal*I to isolate a 4.5-kbp *Eco*RI–*Sal*I fragment (fragment F). Fragments E and F were ligated together into pRD023 previously digested with *Eco*RI and *Hin*dIII, to generate plasmid pRD029 (Fig. 2C). In order to introduce a *Not*I site upstream from the IBDV VP2 start codon and a second *Not*I site downstream from the IBDV VP2 stop codon, a PCR was done using primers GB011 5′ GCTGCGGCCGCTCTAGAACTCGTCGATCGCAGCGATGACAAAC 3′ and GB012 5′ GCTGCGGCCGCTCTAGATCAAGCTTACCTCCTTATAGCC 3′ and pIBDVA01 as template. The 1.4-kbp PCR fragment was digested with *Not*I and cloned into plasmid pRD029 previously digested with *Not*I and treated with alkaline phosphatase, to generate donor plasmid pEL025 (Fig. 2D). This plasmid contains the 5′ and 3′ HVT gI flanking sequences surrounding the VP2 gene of IBDV under the control of the human cytomegalovirus immediate early (HCMV-IE) promoter. The HCMV-IE promoter sequence used in pEL025 corresponds to a 594-bp *Tha*I fragment extending from nucleotide −522 to nucleotide +72 relative to the transcription initiation site (Boshart *et al.*, 1985). Note that the HCMV-IE promoter region used in our construct contains the SV40 late viral protein gene 16s/19s splice donor and acceptor signals. This sequence is located in pEL025 between the HCMV-IE promoter and the IBDV VP2 gene.

Transfection and isolation of HVT recombinants

Primary CEFs seeded at 3 × 10^6 cells per 60-mm dish, were cotransfected with 1 μg of linearized plasmid DNA and 5 μg of total HVT (19th passage since first isolation of HVT on turkey; Witter *et al.*, 1970)-infected cell DNA using Lipofectin according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD). Three days after cotransfection, cells were harvested, pooled with fresh secondary CEFs, and plated on two 60-mm dishes. After 3 days, when plaques were visible, cells were harvested and used to infect microtiter plates by limiting dilution. Recombinant HVT plaques expressing the VP2 protein were identified by an indirect immunofluorescence assay using anti-VP2 monoclonal antibody (1C6; Rhône Mérieux). Monoclonal antibody 1C6 was obtained after immunization of mice with the Lukert strain of IBDV (Lukert, 1985). Characterization of 1C6 was performed using virus neutralization test and the VP2 specificity was identified by radioimmunoprecipitation analysis. Purification of recombinant viruses was obtained by successive rounds of limiting dilution and single plaque isolation. Southern blot technique was used to confirm that recombination occurred as predicted at the targeted site and that recombinant HVT population was wild-type free.

Transient expression assay

This test was done for the *in vitro* evaluation of promoter activities using plasmids containing the *lacZ* gene under the control of different promoters. For each assay, 4×10^6 primary CEFs were transfected with 3 μg of plasmid DNA using 100 μg of LipofectAMINE as described by the manufacturer (Gibco BRL). Transfection was done in double. Twenty-four hours later, one transfection was infected with 0.8×10^6 CEFs derived from a fresh HVT culture. The other transfection was not infected. One day after HVT infection, cells were harvested and extracts were prepared and assayed for β-galactosidase activity as described by MacGregor *et al.* (1991) using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. β-Galactosidase activity was expressed in units per milligram of total protein extract in such a way that 1 U is equivalent to conversion of 1 nmol of ONPG/min at 37°.

IBDV challenge experiment

Two different experiments were done. In the first experiment, SPF chicks of 1 day of age were vaccinated intramuscularly (im) with different doses of vHVT001 or parental HVT. Several birds of each group were sample bled at Day 21 postvaccination for bursal disease seroneutralization (SN) antibody titer determination as described previously (Jackwood *et al.*, 1982). Birds were challenged 21 days postvaccination by ocular route with $10^{2.5}$ mean embryo infectious dose of IBDV 52/70 strain. Four days after challenge, surviving birds were euthanized by intracardiac injection of barbiturates and bursae were dissected. Protection from bursal disease was evaluated by bursal gross lesion examination. In the second experiment, SPF chicks of 1 day of age were vaccinated im with different doses of vHVT001, vHVT002, or parental HVT or with an inactivated vaccine, Gumboriffa (Rhône Mérieux). SN tests and challenge were done as in the first experiment. Eleven days after challenge, surviving birds were euthanized and bursae were dissected. Protection from bursal disease was evaluated by bursal gross lesion examination and bursal/body weight ratio calculation.

MDV challenge experiment

SPF chicks of 1 day of age were vaccinated im with different doses of recombinant viruses or parental HVT. Seven days postvaccination, birds were challenged intraperitoneally (ip) with 50 μl of blood collected from chickens infected with MDV RB1B strain. This amount of blood was sufficient to kill 100% of unvaccinated chickens. Birds were observed daily. Dying animals were euthanized by intracardiac injection of barbiturates and tissues were examined for gross tumors. Ten weeks postchallenge, surviving birds were euthanized and necropsied.

Viremia

One-day-old SPF chickens were inoculated im with different doses of recombinant viruses or parental HVT. Buffy coat cells (BCC) were prepared from blood samples obtained by intracardiac puncture at 7, 11, 14, and 21 days postvaccination. Approximately 5 ml of heparinized blood was centrifuged at 30 *g* for 15 min at room temperature. BCC were recovered, washed in PBS, resuspended in M199 medium (Gibco BRL), and counted. Cocultivations were done by seeding 2×10^6 BCC onto 60-mm tissue culture dishes plated the day before with 3×10^6 primary CEFs. Virus plaques were counted 4 days later.

All animal protocols were approved by the Institutional Animal Care and Use Committee of Rhône Mérieux Laboratoire de Lyon.

RESULTS

Construction of recombinant HVT containing the IBDV VP2 gene at RR2 locus (vHVT001)

Construction of donor plasmid pGH010 used to isolate the RR2 deletion mutant is outlined in Fig. 1 and described under Materials and Methods. This plasmid contains: (i) the 766-bp sequence located 6 bp upstream from the RR2 start codon as 5′ flanking sequence; (ii) the IBDV VP2 coding sequence consisting of the first 453 amino acids of IBDV VP2–VP4–VP3 polyprotein; and (iii) the 1932-bp sequence located 29 bp downstream from the RR2 stop codon as 3′ flanking sequence. The HVT sequences of HSV-1 RR1 and HSV-1 RR2 homologous genes are available on EMBL/GenBank under Accession No. L40429. Plasmid pGH010 was cotransfected with infectious HVT DNA in CEFs. Recombinant HVT clones expressing the VP2 protein were identified by indirect immunofluorescence using an anti-VP2 monoclonal antibody (1C6) and were plaque-purified. After five rounds of purification, one recombinant HVT was selected and designated vHVT001. The recombinant genomic structure was confirmed by Southern blot analysis and by restriction enzyme map comparison with the parental HVT (data not shown).

Construction of recombinant HVT containing the IBDV VP2 gene at gI locus (vHVT002)

The pEL025 construction (Fig. 2) resulted in a deletion of 1036 bp of the 1068 bp of the HVT gI gene (Zelnik *et al.*, 1993). In this donor plasmid, the IBDV VP2 gene expression is under the control of the HCMV-IE enhancer/promoter. Plasmid pEL025 was cotransfected with infectious HVT DNA and a recombinant HVT clone was isolated after three rounds of purification and characterized by Southern blot analysis as above (data not shown). This clone was designated vHVT002.

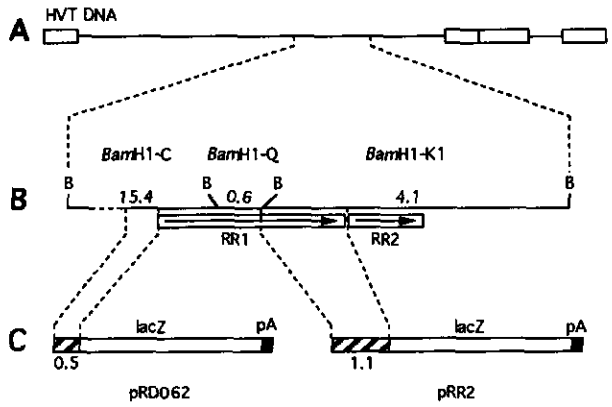

FIG. 3. Description of plasmids used in transient expression assay. (A) Structure of the HVT genome showing location of the *Bam*H1-C, *Bam*H1-Q, and *Bam*H1-K1 fragments. (B) Map of encoded open reading frames in the region of interest. (C) Structure of plasmids pRD062 and pRR2 showing HVT promoter regions (hatched bars), *lacZ* gene (open bars), and SV40 late gene polyadenylation signal (pA, closed bars). Fragment sizes are in kbp. Abbreviations: B, *Bam*HI.

Comparison of the promoters used in vHVT001 and vHVT002 in a transient expression assay

This assay was done with four different plasmids: pCMVβ (Clontech), pRR2, pRD062, and pRD017. Plasmid pCMVβ contains the *lacZ* gene under the control of HCMV-IE promoter region (Fig. 2C). The three other plasmids were constructed from pCMVβ and only promoter regions are different in those plasmids. Plasmid pRR2 contains the 1.1-kbp sequence located upstream from the HVT RR2 start codon as RR2 promoter element. Plasmid pRD062 contains the 0.5-kbp sequence located upstream from the HVT RR1 start codon as RR1 promoter element (Fig. 3). The detection in other herpesviruses of two 3′-coterminal transcripts encoding RR1 and RR2 subunits (McLauchlan and Clements, 1983; De Wind *et al.*, 1994), suggests that expression of the VP2 gene in vHVT001 can be driven by the RR1 and/or RR2 promoter(s). These two promoters were therefore tested in our assay. Plasmid pRD017, without promoter element upstream of the *lacZ* gene, was used as negative control. CEFs were transfected with those plasmids. Transfected cells were infected or not with HVT, and cell extracts were prepared and tested for β-galactosidase activity (Table 1). HVT RR1 and HVT RR2 promoter activities were detectable only when transfected cells were infected and were respectively 100 and 1600 times lesser than HCMV-IE promoter activity. In the two experiments, these ratios were conserved, in spite of activity variations. Note that HCMV-IE promoter activity was increased 2.5-fold in presence of HVT infection.

Evaluation of protection against IBDV challenge

Two experiments were done to evaluate the protection of chickens vaccinated with vHVT001 or vHVT002 against a Gumboro disease challenge. The results are presented in Table 2. In the first experiment, only vHVT001 was tested and a similar level of protection was obtained with 10^5 and 10^4 PFU doses (50 and 33%, respectively), but no protection was observed with a dose of 10^3 PFU. In the second experiment, surviving birds were euthanized for bursa examination 7 days later compared to the first experiment, and bursal/body weight ratios were calculated. No protection was induced by vHVT001. In contrast, vHVT002-vaccinated chickens displayed 100 and 60% protection with 10^5 and 10^4 PFU doses, respectively.

To examine vHVT001 and vHVT002 ability to induce an anti-VP2 humoral response, chickens were tested 21 days postvaccination for the presence of antibodies by SN test (Table 2). Results showed that vHVT001 (especially in the first experiment) and vHVT002 were able to induce neutralizing antibodies in vaccinated chicken sera. However, SN antibody titers obtained from recombinant HVT-vaccinated chickens were at least lower by 2 $\log_{10}$ when compared to titers of chickens vaccinated with the inactivated vaccine. In addition, neutralizing antibodies were detected in all protected animals (data not shown).

Since experiment 1 and experiment 2 challenges are comparable (identical mortality rate in both groups of nonvaccinated control chickens), the observed differences in protection induced by vHVT001 cannot be due to a difference in the challenge level. However, analysis of mortality rates for the vHVT001 groups in experiments 1 and 2 showed significant differences. Moreover, the numbers of chickens displaying measurable SN titers in those groups are also different although chickens received theoretically the same amount of recombinant virus in both experiments. These data point out a possible problem that may have occurred during vaccination with vHVT001 in the second experiment, but the actual reason is not known.

Evaluation of protection against Marek's disease challenge

Protection against Marek's disease virus challenge of chickens vaccinated with recombinant HVT viruses was investigated. One-day-old chickens were vaccinated im with 10^2 and 10^3 PFU of vHVT001, vHVT002, or parental HVT and were challenged 7 days later with the virulent RB1B strain of MDV (Table 3). Protection levels obtained with the highest dose (10^3 PFU) of vHVT001 or vHVT002 were low (6 and 10%, respectively), whereas chickens vaccinated with the same dose of parental HVT displayed 84% protection. These results indicate that protection efficacy of both recombinant viruses is strongly reduced.

Viremia induced by vHVT001 and vHVT002 in chickens

Buffy coat cells of chickens vaccinated with 10^3 or 10^4 PFU dose of recombinant or parental HVT were prepared

TABLE 1

Activity of Promoters Used for vHVT001 and vHVT002

Plasmid[a]	Promoter	β-Gal activity (U/mg)[b]			
		Without HVT infection		With HVT infection	
		Expt 1	Expt 2	Expt 1	Expt 2
None[c]	—	0	0	0	0
pRD017	—	<1[d]	<1	<1	<1
pCMVβ	HCMV-IE	4200	2200	11,200	5600
pRD062	HVT RR1	<1	<1	114	56
pRR2	HVT RR2	<1	<1	8	3

[a] CEFs were transfected with different plasmid DNA as described under Materials and Methods.

[b] β-Galactosidase activities of HVT-infected or uninfected cell extracts were measured as described under Materials and Methods. Activities are expressed in units per milligram of protein extract.

[c] Transfection was done without plasmid DNA. This sample was used as blank for optic density measurement.

[d] β-Galactosidase activity was lower than 1 U/mg (threshold of detection).

at 7, 11, 14, and 21 days after vaccination (Table 4). Viremia in vHVT001-vaccinated chickens was detected at 7 and 11 days with a decrease of the viremic chickens/total number of chickens ratio at Day 11. In contrast, no viremia could be detected in vHVT002-inoculated chickens. Parental HVT was isolated from all chickens and no significant dose effect was observed. These results indicate that *in vivo* replication of both RR2- or gI-deleted viruses is impaired.

DISCUSSION

Results from the IBDV challenge experiments showed that expression of the VP2 protein by an HVT recombi-

TABLE 2

Protection of SPF Chickens Vaccinated with HVT, Recombinant HVT viruses, or Gumboriffa Vaccine (Rhône Mérieux) at 1 Day of Age and Challenged at 21 Days of Age by Ocular Route with 52/70 Strain of IBDV

Expt	Vaccine	Dose[a]	Dead birds	Bursal lesions[b]	Protection[c]	Bursal/body weight ratio[d]	SN[e]
1	vHVT001	10^5	0/12	6/12	50%	ND[f]	3/5
		10^4	2/12	6/12	33%	ND	2/5
		10^3	1/12	11/12	0%	ND	1/5
	None[g]	—	7/12	5/12	0%	ND	0/5
2	vHVT001	10^5	3/10	7/10	0%	0.23 ± 0.18	1/5
		10^4	6/10	4/10	0%	0.20 ± 0.16	0/5
		10^3	4/9	5/9	0%	0.14 ± 0.03	0/5
	vHVT002	10^5	0/9	0/9	100%	0.67 ± 0.11	5/5
		10^4	0/10	4/10	60%	0.20 ± 0.05[h] 0.73 ± 0.22	3/5
		10^3	4/9	5/9	0%	0.22 ± 0.13	0/5
	HVT	10^5	7/10	3/10	0%	0.32 ± 0.23	0/5
	None	—	5/10	5/10	0%	0.21 ± 0.11	0/5
	Gumboriffa	0.3 ml	0/10	0/10	100%	0.67 ± 0.12	5/5

[a] One-day-old chickens were vaccinated intramuscularly with the indicated number of PFU of cell-associated virus contained in 0.2 ml. Gumboriffa (inactivated IBDV vaccine, Rhône Mérieux)-immunized birds received 0.3 ml of vaccine intramuscularly.

[b] Number of surviving birds with bursal lesions/total number in group.

[c] Number of surviving birds without bursal lesion/total number in group expressed as a percentage.

[d] Mean of individual bursal/body weight ratio expressed in % ± standard deviation.

[e] Five birds of each group were sample bled 21 days postvaccination for bursal disease seroneutralization (SN) antibody titer determination. Results are expressed in number of birds with detectable SN antibody titer/total number tested.

[f] Not determined.

[g] Unvaccinated challenge controls.

[h] Bursal/body weight ratio of birds with bursal lesions followed by bursal/body weight ratio of protected birds.

TABLE 3

Protection of SPF Chickens Vaccinated with HVT or Recombinant HVT Vaccines at 1 Day of Age and Challenged Intraperitoneally 7 Days Later with the RB1B Strain of MDV

Vaccine	Dose[a]	Dead birds	Gross lesions[b]	Protection[c]
vHVT001	10^3	15/18	2/18	8%
	10^2	16/20	2/20	10%
vHVT002	10^3	16/20	2/20	10%
	10^2	19/19	0/19	0%
HVT	10^3	3/19	0/19	84%
	10^2	7/19	1/17	53%
None[d]	—	25/25	0/25	0%

[a] Chickens were vaccinated intramuscularly with the indicated number of PFU of cell-associated virus.
[b] Number of surviving birds with gross tumors/total number in group.
[c] Number of surviving birds without gross tumor/total number in group expressed as a percentage.
[d] Unvaccinated challenge controls.

nant virus (vHVT002) was able to induce production of anti-VP2 neutralizing antibodies in chickens and was able to protect all vaccinated chickens against mortality and against damage to the bursa of Fabricius. Full protection against IBDV challenge was also obtained by others, using a recombinant fowlpox virus expressing the VP2 antigen (Heine and Boyle, 1993). This protection was achieved by vaccination of chickens at 21 days of age and by revaccination 14 days later. Our report is the first which describes 100% protection against IBDV challenge after vaccination of 1-day-old chickens with a single injection of a recombinant virus. However, this full protection was obtained using a relatively high dose (10^5 PFU/chicken) of recombinant virus which impedes its use for mass vaccination by the poultry industry for economic reasons.

Only partial protection against IBDV was induced by vHVT001. The difference in results obtained between vHVT001 and vHVT002 could be due to a difference in promoter strength for driving the transcription of the foreign gene. No exogenous promoter is inserted upstream of the VP2 coding sequence in vHVT001 and transcription of VP2 is probably initiated by the promoter of the deleted gene (RR2) and could be also initiated by that of the upstream gene (RR1). In the case of vHVT002, an exogenous promoter (HCMV-IE) is inserted upstream of the VP2 gene. *In vitro* comparison of those promoters using a transient expression assay showed a very large difference in activity. HVT RR1 and HVT RR2 promoter activities were respectively 100 and 1600 times smaller than that of HCMV-IE promoter. Although it is not known if similar differences exist *in vivo*, those results could explain the differences in protection induced by vHVT001 and vHVT002 against the IBDV virulent challenge.

The isolation of vHVT001 and vHVT002 recombinant viruses indicated that the deleted genes (HSV-1 ribonucleotide reductase small subunit homolog in vHVT001 and HSV-1 gI homolog in vHVT002) were not required for growth of HVT in chicken embryo fibroblasts. Those results are consistent with previous reports which dem-

TABLE 4

HVT Viremia Levels after Intramuscular Inoculation of One-Day-Old Chickens with HVT or Recombinant HVT

		Average viremia (PFU/10^6 BCC ± SD)[b]			
Vaccine	Dose[a]	Day 7	Day 11	Day 14	Day 21
vHVT001	10^4	3.2 ± 3.0 (5/5)	0.2 ± 0.4 (1/5)	0[c] (0/5)	0 (0/5)
	10^3	1.7 ± 2.7 (2/5)	0.1 ± 0.2 (1/5)	0 (0/5)	0 (0/5)
vHVT002	10^4	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)
	10^3	0 (0/5)	0 (0/5)	0 (0/5)	0 (0/5)
HVT	10^4	23.3 ± 14.8 (4/4)	26.8 ± 11.7 (4/4)	11.8 ± 6.6 (4/4)	12.8 ± 7.2 (4/4)
	10^3	18.0 ± 16.9 (4/4)	17.3 ± 5.1 (4/4)	17.8 ± 8.8 (4/4)	12.4 ± 5.6 (4/4)
None[d]	—	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)

[a] Chickens were inoculated intramuscularly with the indicated number of PFU of cell-associated virus.
[b] Blood samples were obtained by cardiac puncture 7, 11, 14, and 21 days postinoculation and buffy coat cells (BCC) were isolated for cocultivation with CEFs. Average viremia was calculated using five chickens per group (number of birds with detectable viremia/number of tested birds).
[c] No viremia was detected in any bird of the group (viremia <0.5 PFU/10^6 BCC for each bird).
[d] Noninoculated control chickens.

onstrated that HSV-1 RR2 (Preston *et al.*, 1988), HSV-1 gI (Balan *et al.*, 1994), and pseudorabies virus (PRV) gI (gp63) (Petrovskis *et al.*, 1986) were not essential for herpesvirus replication in cell culture. However, the viremia and the protection against MDV challenge observed in chickens inoculated with the RR2-deleted HVT recombinant (vHVT001) were reduced when compared with those observed with the parental virus. This result may be put in parallel with a recently published work that showed that virulence of a PRV mutant carrying a stop codon in the RR2 open reading frame was reduced in a murine model (De Wind *et al.*, 1994). In the case of the gI-deleted HVT recombinant (vHVT002), the absence of detectable viremia and the reduced ability to protect chickens against MDV challenge suggest either a poor or a lack of *in vivo* replication. Such impairment during *in vivo* replication is also illustrated by the strict dose dependence of the protective effect of this virus against IBDV challenge (drop from 100 to 0% for 10^5 and 10^3 PFU doses, respectively). A similar limitation of *in vivo* replication has been observed with gI-deleted mutants obtained from PRV (Kimman *et al.*, 1992) and from HSV-1 (Balan *et al.*, 1994). In addition, it has been demonstrated that gI, through the gI–gE complex, plays an important role for the spread of HSV-1 from infected to uninfected cells by cell contact (Balan *et al.*, 1994). In the case of HVT which displays a cell-associated nature (Witter *et al.*, 1970), the effect of a gI deletion on viral amplification could be even more dramatic. In addition, presence of unknown mutations in vHVT002 unrelated to the homologous recombination event, which could have been picked up during the transfection or purification process, cannot be excluded. Such undetected mutations could enhance the consequences of the gI deletion effect on vHVT002 *in vivo* replication. This remark is also valid for vHVT001.

Comparison of our two recombinant HVT viruses showed that the targeted site for insertion of the foreign gene and the promoter used to drive the expression of this gene were two important parameters. Additional studies on recombinant HVT viruses expressing the IBDV VP2 antigen are needed to improve the vaccine. First, new insertion sites which do not impair the *in vivo* replication rate of the recombinant virus should be tested such as US10 (Morgan *et al.*, 1992, 1993) or US2 (Parcells *et al.*, 1994). Second, the effect of maternal anti-IBDV and anti-MDV antibodies on vaccination with recombinant HVT viruses should be determined. Third, protection against a nonhomologous challenge, using variant or highly virulent strains of IBDV, should be studied to determine whether VP2 (strain 52/70) can still provide protection.

Results of protection against an IBDV challenge (this paper), and against an NDV challenge (Morgan *et al.*, 1992, 1993), using specific HVT recombinant viruses, demonstrate the potential use of HVT as a viral vector for expressing protective immunogens in chickens.

ACKNOWLEDGMENTS

We thank Patrick Mérut, Bernard Morel, and Jacques-Olivier Dru for their excellent technical assistance. These studies were supported by the EEC BRIDGE Program BIOT CT-900173.

REFERENCES

Azad, A. A., Barrett, S. A., and Fahey, K. J. (1985). The characterization and molecular cloning of the double-stranded RNA genome of an Australian strain of infectious bursal disease virus. *Virology* **143,** 35–44.

Azad, A. A., Jagadish, M. N., Brown, M. A., and Hudson, P. J. (1987). Deletion mapping and expression in *Escherichia coli* of the large genomic segment of a birnavirus. *Virology* **161,** 145–152.

Azad, A. A., McKern, N. M., Macreadie, I. G., Failla, P., Heine, H.-G., Chapman, A., Ward, C. W., and Fahey, K. J. (1991). Physicochemical and immunological characterization of recombinant host-protective antigen (VP2) of infectious bursal disease virus. *Vaccine* **9,** 715–722.

Balan, P., Davis-Poynter, N., Bell, S., Atkinson, H., Browne, H., and Minson, T. (1994). An analysis of the *in vitro* and *in vivo* phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI or the putative gJ. *J. Gen. Virol.* **75,** 1245–1258.

Bayliss, C. D., Spies, U., Shaw, K., Peters, R. W., Papageorgiou, A., Müller, H., and Boursnell, M. E. G. (1990). A comparison of the sequences of segment A of four infectious bursal disease virus strains and identification of a variable region in VP2. *J. Gen. Virol.* **71,** 1303–1312.

Bayliss, C. D., Peters, R. W., Cook, J. K. A., Reece, R. L., Howes, K., Binns, M. N., and Boursnell, M. E. G. (1991). A recombinant fowlpox virus that expresses the VP2 antigen of infectious bursal disease virus induces protection against mortality caused by the virus. *Arch. Virol.* **120,** 193–205.

Becht, H., Müller, H., and Müller, H. K. (1988). Comparative studies on structural and antigenic properties of two serotypes of infectious bursal disease virus. *J. Gen. Virol.* **69,** 631–640.

Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985). A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* **41,** 521–530.

Bygrave, A. C., and Faragher, J. T. (1970). Mortality associated with Gumboro disease. *Vet. Rec.* **86,** 758–759.

De Wind, N., Peeters, B. P. H., Zijderveld, A., Gielkens, A. L. J., Berns, A. J. M., and Kimman, T. G. (1994). Mutagenesis and characterization of a 41-kilobase-pair region of the pseudorabies virus genome: Transcription map, search for virulence genes, and comparison with homologs of herpes simplex virus type 1. *Virology* **200,** 784–790.

Dobos, P., Hill, B. J., Hallett, R., Kells, D. T. C., Becht, H., and Tenninges, D. (1979). Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. *J. Virol.* **32,** 593–605.

Fahey, K. J., Erny, K., and Crooks, J. (1989). A conformational immunogen on VP-2 of infectious bursal disease virus that induces virus-neutralizing antibodies that passively protect chickens. *J. Gen. Virol.* **70,** 1473–1481.

Fahey, K. J., McWaters, P., Brown, M. A., Erny, K., Murphy, V. J., and Hewish, D. R. (1991). Virus-neutralizing and passively protective monoclonal antibodies to infectious bursal disease virus of chickens. *Avian Dis.* **35,** 365–373.

Gubler, U., and Hoffman, B. J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* **25,** 263–269.

Heine, H.-G., and Boyle, D. B. (1993). Infectious bursal disease virus

structural protein VP2 expressed by a fowlpox virus recombinant confers protection against disease in chickens. *Arch. Virol.* **131,** 277–292.

Igarashi, T., Takahashi, M., Donovan, J., Jessip, J., Smith, M., Hirai, K., Tanaka, A., and Nonoyama, M. (1987). Restriction enzyme map of herpesvirus of turkey DNA and its collinear relationship with Marek's disease virus DNA. *Virology* **157,** 351–358.

Jackwood, D. J., Saif, Y. M., Moorhead, P. D., and Dearth, R. N. (1982). Infectious bursal disease virus and alcaligenes faecalis infections in turkeys. *Avian Dis.* **26,** 365–374.

Kibenge, F. S. B., Dhillon, A. S., and Russell, R. G. (1988). Biochemistry and immunology of infectious bursal disease virus. *J. Gen. Virol.* **69,** 1757–1775.

Kimman, T. G., Pol, J. M. A., De Wind, N., Oei-Lie, N., Berns, A. J. M., and Gielkens, A. L. J. (1992). Role of different genes in the virulence and pathogenesis of Aujeszky's disease virus. *Vet. Microbiol.* **33,** 45–52.

Kunkel, T. A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82,** 488–492.

Lee, Y. S., Tanaka, A., Lau, R. Y., Nonoyama, M., and Rabin, H. (1980). Comparative studies of herpesvirus papio (baboon herpesvirus) DNA and Epstein–Barr virus DNA. *J. Gen. Virol.* **51,** 245–253.

Lukert, P. D. (1985). Serotyping recent isolates of infectious bursal disease virus. Abstr. 123rd Am. Vet. Med. Assoc. Meeting, p. 182.

MacGregor, G. R., Nolan, G. P., Fiering, S., Roederer, M., and Herzenberg, L. A. (1991). Use of *E. coli lacZ* (β-galactosidase) as a reporter gene. *In* "Methods in Molecular Biology," Vol. 7, "Gene Transfer and Expression Protocols" (E. J. Murray, Ed.), pp. 217–235. Humana Press, Clifton, NJ.

Macreadie, I. G., Vaughan, P. R., Chapman, A. J., McKern, N. M., Jagadish, M. N., Heine, H.-G., Ward, C. W., Fahey, K. J., and Azad, A. A. (1990). Passive protection against infectious bursal disease virus by viral VP2 expressed in yeast. *Vaccine* **8,** 549–552.

McLauchlan, J., and Clements, J. B. (1983). Organization of the herpes simplex type 1 transcription unit encoding two early proteins with molecular weights of 140,000 and 40,000. *J. Gen. Virol.* **64,** 997–1006.

Morgan, R. W., Cantello, J. L., and McDermott, C. H. (1990). Transfection of chicken embryo fibroblasts with Marek's disease virus DNA. *Avian Dis.* **34,** 345–351.

Morgan, R. W., Gelb, J. Jr., Schreurs, C. S., Lütticken, D., Rosenberger, J. K., and Sondermeijer, P. J. A. (1992). Protection of chickens from Newcastle and Marek's diseases with a recombinant herpesvirus of turkeys vaccine expressing the Newcastle disease virus fusion protein. *Avian Dis.* **36,** 858–870.

Morgan, R. W., Gelb, J. Jr., Pope, C. R., and Sondermeijer, P. J. A. (1993). Efficacy in chickens of a herpesvirus of turkeys recombinant vaccine containing the fusion gene of Newcastle disease virus: Onset of protection and effect of maternal antibodies. *Avian Dis.* **37,** 1032–1040.

Mundt, E., Beyer, J., and Müller, H. (1995). Identification of a novel viral protein in infectious bursal disease virus-infected cells. *J. Gen. Virol.* **76,** 437–443.

Öppling, V., Müller, H., and Becht, H. (1991a). Heterogeneity of the antigenic site responsible for the induction of neutralizing antibodies in infectious bursal disease virus. *Arch. Virol.* **119,** 211–223.

Öppling, V., Müller, H., and Becht, H. (1991b). The structural polypeptide VP3 of infectious bursal disease virus carries group- and serotype-specific epitopes. *J. Gen. Virol.* **72,** 2275–2278.

Parcells, M. S., Anderson, A. S., Cantello, J. L., and Morgan, R. W. (1994). Characterization of Marek's disease virus insertion and deletion mutants that lack US1 (ICP22 homolog), US10, and/or US2 and neighboring short-component open reading frames. *J. Virol.* **68,** 8239–8253.

Petrovskis, E. A., Timmins, J. G., Gierman, T. M., and Post, L. E. (1986). Deletions in vaccine strains of pseudorabies virus and their effect on synthesis of glycoprotein gp63. *J. Virol.* **60,** 1166–1169.

Preston, V. G., Darling, A. J., and McDougall, I. M. (1988). The herpes simplex virus type 1 temperature-sensitive mutant ts1222 has a single base pair deletion in the small subunit of ribonucleotide reductase. *Virology* **167,** 458–467.

Reddy, S. K., Silim, A., and Ratcliffe, M. J. H. (1992). Biological roles of the major capsid proteins and relationships between the two existing serotypes of infectious bursal disease virus. *Arch. Virol.* **127,** 209–222.

Rosenberger, J. K., and Cloud, S. (1985). Isolation and characterization of variant infectious bursal disease viruses. Abstr. 123rd Am. Vet. Med. Assoc. Meeting, p. 357.

Ross, L. J. N., Basarab, O., Walker, D. J., and Whitby, B. (1975). Serological relationship between a pathogenic strain of Marek's disease virus, its attenuated derivative and herpesvirus of turkeys. *J. Gen. Virol.* **28,** 37–47.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schat, K. A., Calnek, B. W., and Fabricant, J. (1982). Characterization of two highly oncogenic strains of Marek's disease virus. *Avian Pathol.* **11,** 593–605.

Snyder, D. B., Lana, D. P., Savage, P. K., Yancey, F. S., Mengel, S. A., and Marquardt, W. W. (1988). Differentiation of infectious bursal disease viruses directly from infected tissues with neutralizing monoclonal antibodies: Evidence of a major antigenic shift in recent field isolates. *Avian Dis.* **32,** 535–539.

Snyder, D. B., Vakharia, V. N., and Savage, P. K. (1992). Naturally occurring neutralizing monoclonal antibody escape variants define the epidemiology of infectious bursal disease virus in the United States. *Arch. Virol.* **127,** 89–101.

Spies, U., Müller, H., and Becht, H. (1987). Properties of RNA polymerase activity associated with infectious bursal disease virus and characterization of its reaction products. *Virus Res.* **8,** 127–140.

Vakharia, V. N., Snyder, D. B., Lütticken, D., Mengel-Whereat, S. A., Savage, P. K., Edwards, G. H., and Goodwin, M. A. (1994). Active and passive protection against variant and classic infectious bursal disease virus strains induced by baculovirus-expressed structural proteins. *Vaccine* **12,** 452–456.

Van den Berg, T. P., Gonze, M., and Meulemans, G. (1991). Acute infectious bursal disease in poultry: Isolation and characterisation of a highly virulent strain. *Avian Pathol.* **20,** 133–143.

Witter, R. L. (1985). Principles of vaccination. *In* "Marek's Disease" (L. N. Payne, Ed.), pp. 203–250. Nijhoff, Boston.

Witter, R. L., Nazerian, K., Purchase, H. G., and Burgoyne, G. H. (1970). Isolation from turkeys of a cell-associated herpesvirus antigenically related to Marek's disease virus. *Am. J. Vet. Res.* **31,** 525–538.

Zelnik, V., Darteil, R., Audonnet, J. C., Smith, G. D., Riviere, M., Pastorek, J., and Ross, L. J. N. (1993). The complete sequence and gene organization of the short unique region of herpesvirus of turkeys. *J. Gen. Virol.* **74,** 2151–2162.